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Research review

In vivo modeling of biofilm-infected wounds: A review

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ABSTRACT

Chronic wounds continue to represent a difficult and complex problem for both patients and healthcare providers. Bacterial biofilms represent a critical component of nonhealing wounds, utilizing several different mechanisms to inhibit innate inflammatory pathways and resist traditional therapeutics. Although *in vitro* biofilm systems have been well described and studied, understanding the intricacies of wound biofilm pathology requires appropriate *in vivo* models to understand the interactions between bacteria and host. In an effort to clarify the available literature, this review describes and critically evaluates all of the *in vivo* wound biofilm models currently published to date, including model advantages and clinical applicability. We will also address the need for continued therapeutic development and testing using these currently available *in vivo* models.

1. Introduction

The management and treatment of chronic wounds represents a significant burden on both millions of patients and on the healthcare system [1–4]. With the majority of research historically aimed at understanding lifestyle and genetically dependent contributors to wound pathogenesis, the impact of bacterial biofilms on wound healing has only recently gained interest within the scientific community [5–19]. The inherent defense and survival mechanisms of microbial biofilms, including avoidance of host inflammatory cells [20,21], resistance to antibiotics [22–24], and dynamic cell cell communication pathways [11,25] makes them a remarkably durable constituent of the nonhealing wound. Until recently, the majority of studies within this field have been performed using *in vitro* systems [26–29], restricting the adaptability of these findings to clinical situations. Several *in vivo* wound biofilm models have been published within the last few years [30–39], each bringing distinct strengths and weaknesses in their attempt to simulate human chronic wounds. In this review, we discuss the clinical relevance of *in vivo* models of biofilm infected wounds, as well as critically evaluate those models that have been published to date. Although a variety of models have been published examining foreign body infections as well as osteomyelitis, we focus specifically in this review on

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models that have a direct clinical relevance to an open cutaneous wound.

2. Significance of biofilm in wounds

Chronic wounds are a significant and growing problem in healthcare today. Healthcare costs associated with chronic wound management and treatment in the United States were estimated to be upwards of $20 billion $25 billion annually in 2008 [1–4,40–49]. However, these costs do not include the impact on patient lifestyle, financial security, and overall well being, which in aggregate represent an immeasurable burden [50–53]. The management of chronic wounds has long relied on the basic principles of debridement, lavage, wound tailored dressings, and antimicrobial therapy when necessary as a systematic means to increase the potential for healing either naturally or through surgical intervention [54–60]. A tremendous investment in research funding and a broader interest on the part of clinicians and scientists has led to significant progress in wound science, but the incidence of chronic wounds and associated complications, such as amputations, continues to grow at an epidemic rate. This is in part due to the growing rate of other chronic diseases that can impact healing within this vulnerable population, most notably obesity, diabetes mellitus, and peripheral vascular disease [61–67], but it likely is also due to an incomplete understanding of the contributing factors that result in a chronic wound.

Bacterial biofilms are a key factor whose importance to wound chronicity and persistence has only recently become widely appreciated [5–19]. A bacterial biofilm can be defined as a complex community of aggregated bacteria embedded within a self secreted matrix of extracellular polymeric substance, or EPS [5,11,13,22] (Fig. 1). This phenotype, thought to be the preferred state of bacteria in their native habitats, is distinct from the free floating, so called "planktonic" bacteria that have been extensively studied and manipulated by microbiologists in laboratory settings for over a century [68]. Biofilms are harbored on surfaces throughout the body such as dental enamel, nasal epithelium, urinary tract mucosa, and endocardium, forming relationships that are either purely commensal (e.g., gastrointestinal mucosa) or pathogenic when established ectopically in tissue that has not developed the immunologic defenses to clear or co exist with the bacterial biofilm (e.g., lung mucosa in association with cystic fibrosis) [10,11,22,69–71]. In addition, infections in foreign materials such as implantable orthopedic and cosmetic prosthetics or intravenous catheters are now thought to be secondary to surface biofilms that form on the implant at the time of insertion or later as a result of hematologic seeding [22,69,72–75]. Human skin represents the largest barrier to outside environmental pathogens in the body, however, its protective mechanisms become compromised on creation of a wound, allowing for exposure to a variety of bacterial flora. The moist, nutritionally supportive microenvironment of the wound bed matrix becomes an ideal setting for formation of bacterial biofilm, creating a destructive and sustainable interaction that impairs host wound healing [13,76]. In most wounds, the inflammatory phase of healing promptly removes devitalized tissue debris and bacteria, thereby enabling the progression into the synthetic and remodeling phases of healing, but in the impaired host (e.g., vascular insufficiency, microvascular disease, diabetes, ischemia reperfusion injury), the uncleared, excessive bacterial burden triggers an elevated, but ineffective, inflammatory response [13,77]. This prolonged, chronic inflammatory state further contributes to the inhibition of wound healing pathways [13].

Understanding the structure and physiology of bacterial biofilms is crucial when discussing its inhibitory effects on wound healing (Fig. 2).The presence of bacterial biofilms in chronic wounds has been confirmed by both imaging and other sophisticated molecular sampling techniques [11,14]. The emergence of molecular techniques over traditional culture dependent methods, which rely on a swab or tissue biopsy, has led to a number of significant findings [15,78,79]. It is now appreciated that the amount of bacteria within a chronic wound is often underestimated when analyzed with traditional microbial assays, particularly in wounds with slow or fastidious growing bacteria [14,80–83]. Furthermore, the majority of chronic wound biofilms have been shown to consist of a mixed population of multiple bacterial species [11,13,18]. Predominant bacteria isolated include various anaerobes, *Serratia*, *Staphylococcus*, and *Pseudomonas*, with one study demonstrating an average of 5.4 species of bacteria in each chronic human wound [84]. In addition to their polybacterial nature, all biofilms (including those in wounds) are inherently robust and resistant to host defense mechanisms. The EPS generated by biofilm state bacteria creates a physical barrier that reduces the efficacy of phagocytosis by inflammatory cells such as neutrophils and macrophages, while also inhibiting activation of the

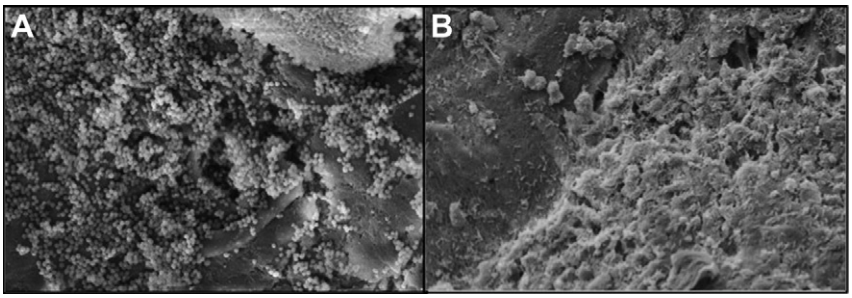

Fig. 1 – Morphology of bacterial biofilm on scanning electron microscopy. Images demonstrate consistency of biofilms formed by *Staphylococcus aureus* (A) and *Pseudomonas aeruginosa* (B) in wounds of the rabbit ear. Note the presence of cocci (A) and rod-shaped (B) bacterial cells within a matrix of extracellular polymeric substance, or EPS. (Magnification: ×2000)

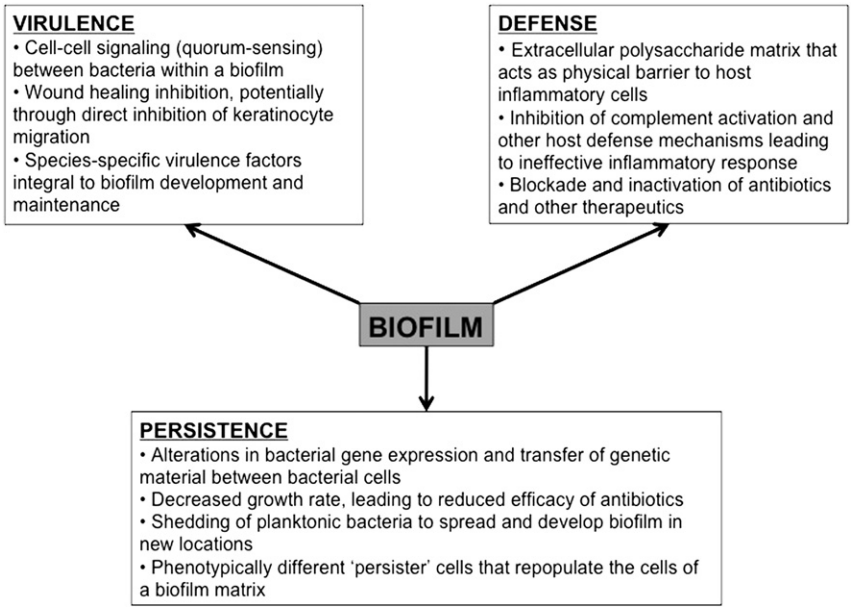

Fig. 2 – Schematic diagram of different characteristics of bacterial biofilm, including mechanisms of virulence, defense, and persistence.

complement cascade [20,21,85]. As stated earlier, this ineffectiveness can result in chronic release of proinflammatory cytokines that can damage nearby tissue [22]. An innate resistance to antimicrobials, potentially up to 1000 times more than their planktonic counterparts, is also characteristic of bacterial biofilm [86]. This has been explained by the inability of antibiotics to penetrate the EPS, and their potential inactivation by alterations within the biofilm microenvironment [22–24]. Furthermore, biofilm bacteria demonstrate a decreased growth rate, leaving them in a sessile state that is less susceptible to most antibiotics, which are typically designed to target rapidly dividing, planktonic bacteria [5,69]. There is also evidence that cell to cell signaling between bacteria within a biofilm, so called quorum sensing, is integral to biofilm development and maintenance [11,25], while alterations in bacterial gene expression and transfer of genetic material between bacteria may enhance inherent survival mechanisms [86]. Finally, the shedding of planktonic bacteria as well as the maintenance of a phenotypically different "persister" cell population are mechanisms for biofilm sustainability and durability within a hostile environment [11,12].

3. Importance of *in vivo* modeling

Although a rapidly growing field of study, there remains an immense gap in basic knowledge about many aspects of biofilm behavior and formation, particularly in the *in vivo* setting. Given the need for new therapeutic approaches in the management of chronic wounds, the importance of understanding the intricacies of biofilm infected wounds cannot be overstated. Research aimed at elucidating the properties of bacterial biofilm and its interactions with the host inflammatory cascade is critical to improving this knowledge base. In particular, the interplay between bacteria and host, represented locally by the wound bed itself, is responsible for some of the defining characteristics of chronic wounds [87,88], and this interplay is not evaluable with *in vitro* models and assays [26–29]. Although such experiments have provided essential knowledge regarding biofilm resistance and survival mechanisms, such as the inhibitory effect of biofilm against cultured human keratinocytes [89], the complexity of the interaction between bacterial biofilms and human wound healing pathways is difficult to extrapolate from *in vitro* biofilm studies.

The lack of adequate *in vivo* models has made it difficult to faithfully model wound biofilms. Human studies are logistically and ethically prohibitive, leaving animal models as the sole practical alternative for systematic investigation and modulation of clinically relevant biofilms. The use of an animal model allows for multiple iterations of experimentation and analysis that cannot be afforded with human research, while allowing for a closer semblance of the biofilm host interaction that is lacking with *in vitro* models. Additionally, the translational nature of *in vivo* modeling provides a more immediate understanding of parallel pathways and mechanisms in human biofilm infected chronic wounds, thus, potentially driving further clinical research. Therefore, an effective *in vivo* model should not only contribute to our scientific and conceptual understanding of biofilm in, but should also provide a foundation and methodology for systematically examining biofilm infected wounds in a precise and quantitative manner.

4. Published *in vivo* models

We believe that an appropriate, consistent, and translatable *in vivo* model of wound biofilm should possess several different, but important, characteristics upon which the strength of a model can be determined (Table 1). A growing

Table 1 – Characteristics necessary for appropriate modeling of *in vivo* wound biofilm.

Characteristic	Apidianakis *et al*	Akiyama *et al*	Rashid *et al*	Davis *et al*	Nakagami *et al*	Simonetti *et al*	Schierle *et al*	Zhao *et al*	Gurjala *et al*
Reproducible and validated (e g , scanning electron microscopy) presence of wound biofilm	No	Yes	No	Yes	No	No	No	No	Yes
Development of biofilm *in vivo* within wounds, similar to human wounds	Yes	Yes	No	Yes	Yes	Yes	No	No	Yes
Consistency in findings, and ease-of-use, across multiple users	Yes	Yes	Yes	Unclear (only one study in literature)	Yes	Yes	Yes	Yes	Yes
Uninfected (control) wound healing with translatability to the healing seen in normal human wounds	No	No	No	No	No	No	Yes	No	Yes
Flexibility to substitute in different bacterial species	Yes	Unclear (only used *S aureus*)	Unclear (only used *P aeruginosa*)	Unclear (only used *S aureus*)	Unclear (only used *P aeruginosa*)	Unclear (only used *S aureus*)	Yes	Unclear (only used *P aeruginosa*)	Yes
Ability to perform data analysis with multiple quantitative and qualitative endpoints, and at several time-points	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ability to introduce and evaluate potential therapeutic agents	Unclear (not previously done)	Yes	Unclear (not previously done)	Yes	Unclear (not previously done)	Yes	Yes	Unclear (not previously done)	Yes

number of groups have designed and utilized novel *in vivo* animal models to understand the role of biofilms within wounds and its direct effects on wound healing [30–39]. The most studied invertebrate model of *in vivo* wound biofilm has been developed in *Drosophila melanogaster*. Although first re ported in the literature by Boman *et al* [90] in 1972, Apidianakis and Rahme [30] recently reviewed the advantages that *D mel anogaster* posseses as a model host for *P aeruginosa*. The authors describe the use of a thoracic or abdominal pin prick to create a wound in the cuticular epithelium and underlying muscle with a needle dipped in a bacterial suspension. This creates a local wound infection, which can then be studied for the early host responses to this bacterial challenge. They also note that several different microbes have been successfully utilized in this model. Although there is a high degree of conservation between mammalian and *D melanogaster* innate immune systems [30], the translatability of invertebrate pin prick wounds to full thickness human chronic wounds remains questionable.

Akiyama *et al* [31] described the first vertebrate model of *in vivo* biofilm, looking at the formation of *Staphylococcus aureus* biofilm in incisional wounds on the backs of mice. Mice were treated with cyclophosphamide prior to inoculation to suppress the strong neutrophil response to *S aureus* that typically occurs in mice. Inoculation was performed with a suspension of *S aureus* in sterile saline at approximately 3.7 $\times$ 10^6 bacterial cells per wound. Qualitative histologic findings demonstrated infiltration of inflammatory cells within *S aureus* clusters over time, while electron microscopy revealed the formation of "membrane like structures" around the bacteria, with minimal invasion by the surrounding inflam matory cells. The authors concluded based on electron microscopy imaging and Ruthenium red staining that these structures were part of a biofilm glycocalyx, indicating that no bacteria were found outside of this matrix by 60 h post inoculation. This work was followed up by examination of the *S aureus* glyocalyx through staining with fluorescein conjugated concanavalin A and confocal laser scanning microscopy in neutropenic and normal mice treated with topical antibiotics [32]. Demonstrating higher bacterial counts in neutropenic animals, the authors concluded that neutro phils may play a crucial role in the host defense against bio film, particularly in helping make bacteria susceptible to antibiotic therapy. However, the effect of this biofilm on the global wound healing response was not evaluated in either of these studies.

In 2000, Rashid *et al* [33] examined the role of the *polyphosphate kinase* gene (PPK) in the virulence and quorum sensing mechanisms of *Pseudomonas aeruginosa* using a previ ously established burned mouse model. Wild type or PPK mutated *P aeruginosa* strains were grown on static or continuous flow cell medias, or injected directly underneath burn wounds at a dose associated with almost 100% mortality by 48 h. Crystal violet staining followed by confocal laser scanning microscopy (was performed to identify and measure the thickness of visualized biofilm, while separate bioassays for different known virulence factors were performed. The authors showed that PPK mutants formed less biofilm on media at 8 and 20 h, with less associated virulence factor expression. *In vitro*, they verified that the wild type strain led

to a greater amount of local and systemic bacterial spread, resulting in higher rates of lethality. However, the majority of the key findings, including identifying the presence of biofilm visually, were performed *in vitro*. Furthermore, similar to Akiyama *et al* [31,32] no assessment of wound healing or host inflammatory mechanisms was performed despite the demonstration of wild type virulence *in vivo*.

In a departure from murine models, a partial thickness, cutaneous porcine wound model was developed by Davis *et al* [34] to study the development of *S aureus* biofilm, including following a topical antimicrobial challenge. Wounds were inoculated with a 10^7 CFU/mL concentration of bacteria by scraping off suspended bacteria from a culture media plate onto each wound, with multiple endpoints including scanning electron, light, and epifluorescence microscopy, as well as bacterial count measurements. To form biofilm, wounds were allowed to proliferate for 48 h following inoculation and Tegaderm (3M Health Care, St. Paul, MN) occlusion to closely model the seeding of bacteria in wounds that occurs clinically. Through different microscopic modalities, they histomorphologically revealed biofilm within wounds at 48 h, with differential effects of topical antimicrobial treatments against planktonic versus biofilm wounds over time. These results were the first *in vivo* evidence of a phenotypic difference between planktonic and biofilm bacteria. However, the use of partial thickness wounds limits a direct correlation to many human chronic wounds, which typically demonstrate full thickness dermal loss as part of their healing impairment. Furthermore, all major endpoints were evaluated relatively early (48 h) after inoculation and, again, the authors did not assess the effects of the bacteria on the healing of the partial thickness wounds.

To simulate a more chronic wound setting, Nakagami *et al* [35] have published the use of a pressure induced ischemic wound model in rats to evaluate quorum sensing mechanisms of *P aeruginosa*. Following inoculation with approximately 10^5 CFU/mL, a known *P aeruginosa* autoinducer that functions to regulate many of the bacteria's virulence factors, was quantified along with wound viable bacterial counts. Histologic analysis showed qualitatively higher levels of tissue destruction and polymorphonuclear leukocytes infiltration in infected wounds, with increasing amounts of the bacterial autoinducer and viable bacterial counts over time. The authors advocated that quantification of such autoinducers may be a useful tool for clinical chronic wound diagnosis. However, similar to previously discussed models, there was no assessment of the wound healing impairment or host inflammatory response that occurs following release of the autoinducer. In addition, visual evidence of biofilm morphology within the ischemic wounds was not presented, which would have helped further validate their model and results.

Simonetti *et al* [36] also addressed quorum sensing pathways using a standard murine wound model, inoculating 5×10^7 CFU/mL of methicillin resistant *S aureus* into wounds. Wounds were treated with different combinations of an adhesive dressing, with or without RNAIII inhibiting peptide (RIP), a quorum sensing inhibitor, and/or the antibiotic teicoplanin. Quantitative measurement of histological wound healing parameters, wound bacterial burden, and vascular endothelial growth factor expression were performed, demonstrating that RIP combined with teicoplanin was found to show the greatest improvements in all measured endpoints as compared to control. However, like many murine, wound healing models, histological wound measurements may be of unclear significance given the wound contracture associated with murine healing.

In an effort to better recapitulate human wound healing, work from our laboratory (Schierle *et al* [37]) utilized an established splinted mouse model to minimize contracture. The importance of minimizing contracture in rodent models of healing is worth emphasizing, as this variable is ignored by most rodent wound healing studies. By minimizing contractures, wounds are allowed to heal by new tissue ingrowth, more akin to human wounds, as opposed to myofibroblast mediated contraction of the loose rodent skin. Treatment of *S aureus* and *Staphylococcus epidermidis* wounds with RIP showed a return of wound healing kinetics to that of control wounds, along with a significant decrease in wound bacterial load. In addition, the use of a biofilm deficient *S aureus* strain also demonstrated improved rates of wound healing over wild type wounds. In contrast, oxacillin treatment of wild type wounds was unable to restore a healing phenotype, presumably due to its inability to eradicate biofilm. These results suggested that the biofilm state of *S aureus* had a direct effect on delaying cutaneous wound healing *in vivo*, and confirmed that therapeutics targeting the biofilm or quorum sensing pathways of skin pathogens may have a clinical role in improving wound healing. It should be noted, however, that no direct visualization of bacterial biofilm and its extracellular matrix were performed, instead relying on Gram stains and quantitative cultures to verify the presence of bacteria presumed to be in a biofilm state in the wounds.

Incorporating another pillar of chronic wound pathogenesis, a diabetic murine model with wound biofilm has been described by Zhao *et al* [38]. Using full thickness circular punch wounds in diabetic strain (db/db) mice, *P aeruginosa* biofilms incubated on agar plates for 72 h were directly transferred onto wounds 48 h post wounding, followed by dressing occlusion for 2 wk. Dressings were then removed and wounds allowed to scab, with basic evaluation of gross and histological healing, measurement of bacterial counts within the scabs and wound beds, transmission electron microscopy of wound scabs to determine morphology and the presence of immune cells. Compared with control wounds, biofilm wounds demonstrated significantly delayed wound healing, as well as inflammatory cell infiltration and tissue changes. Furthermore, when wounds were allowed to scab, the majority of bacteria was found to reside within the scabs of biofilm wounds, with associated neutrophils as seen on transmission electron microscopy. They reported reproducibility and consistency in their results, and thus advocated their model as another *in vivo* approach to study biofilm related delays in chronic wound healing. However, inoculation was performed using the transfer of *in vitro* biofilms on artificial filters. Although this technique is potentially effective, it is not a physiologic representation of how biofilm develops naturally develops within human wounds. In addition, with no evidence of biofilm within the wounds but rather in scabs, it is unclear the applicability of this model to

understanding the direct effects of biofilm on wound healing, particularly of human chronic wounds, the vast majority of which either do not form scabs are not permitted to scab by clinicians.

Most recently, our lab has developed a biofilm adaptation of the rabbit dermal ulcer model [39], an Food and Drug Administration recognized model of wound healing that has been utilized by our lab and others for 20 years [91–99], which we believe embraces a number of the characteristics neces sary for the appropriate modeling of wound biofilm *in vivo* (Table 1). In this model, full thickness, circular punch wounds are made in the ears of New Zealand White rabbits down to cartilage, with multiple identical wounds made in one animal with contralateral, internal controls. Inoculation of wounds is done using culture medium grown bacteria with a measured inoculant concentration of approximately 10^6 bacteria. However, in a significant departure from other published animal models, following *in vivo* proliferation of the inocu lated bacteria, wounds are treated with topical antibiotic. This reduces the presence of active, planktonic phase bacteria, but also by definition leaves behind biofilm phase bacteria, more resistant to antimicrobial challenge due to a protective EPS. This is followed by a combination of an occlusive dressing (Tegaderm) with an underlying antimicrobial (polyhexa methylene biguanide) absorptive gauze pad (AMD Telfa; Tyco Healthcare, Mansfield, MA). This form of wound coverage maintains the predominance of biofilm phase bacteria in two ways. First, the antimicrobial impregnation of the gauze helps limit proliferation of planktonic bacteria. Second, the use of absorptive gauze helps to minimize the formation of seromas from bacterial purulent exudates. Frequent dressing changes are performed at set time points prior to harvest. Beyond quantitative histologic analysis, the model allows for the analysis of several different endpoints after harvest, including the host inflammatory response to biofilm, quantification of wound bacterial burden, and visualization of biofilm morphology and host defense cells through fluorescent and electron microscopy.

By validating the consistent development of a distinct biofilm phenotype within wounds, and demonstrating subsequent effects on wound healing and host inflammatory response, our rabbit model [39] provides several advantages. During wound creation, the removal of dermis, in contrast to partial thickness wounds, more closely models the dermal loss seen in human chronic wounds. Additionally, the majority of human wounds heal through epithelialization and granulation, in contrast to the contracture based healing seen in mice [37]. The underlying cartilage of the rabbit ear serves as a natural splint, preventing healing by contracture, and thus allowing for accurate quantification of epithelial and granulation tissue formation from the periphery of the wound. The creation of multiple wounds, with contralateral internal controls, creates a standardized and high throughput wound model, which avoids cross contamination between wounds by ensuring that each wound within one ear undergoes the same bacterial inoculation and/or treatments. The presence of multiple wounds also does not increase its systemic impact on the host, given their relatively small size and only a localized inflammatory response following bacte rial inoculation [39]. Furthermore, using an absorptive dressing in contrast to the occlusive dressing utilized in other models, prevents the creation of a seroma within the dead space beneath the dressing, which can act as an ideal culture medium for planktonic phase bacteria proliferation. In this setting a mixed planktonic biofilm infection can become a predominantly planktonic, purulent infection, more similar to a superficial abscess than the wound surface biofilms seen in chronic wounds.

The flexibility of the rabbit ear biofilm model also provides a distinct advantage over previously published *in vivo* systems. The rabbit ear allows for the introduction of other classic pathologies associated with chronic wounds, such as ischemia [91]. By modulating blood supply to the ears prior to wounding, a host related variable is introduced into the interaction between wound bed and bacteria, which is diffi cult to appropriately simulate *in vitro* or with other published *in vivo* models. Frequent dressing changes prior to wound harvest, modeling the common clinical management of chronic wounds, allow simultaneous introduction of thera peutic agents or regimens. For example, this model has been used to understand the efficacy of different classical treat ments, such as lavage, silver sulfadiazene, and debridement, on established *P aeruginosa* biofilm wounds [100]. As novel treatments targeting different aspects of biofilm virulence and maintenance are developed, the rabbit ear model provides an established *in vivo* platform for testing these treatments that is directly translatable to the human patient. There are however some drawbacks to the rabbit model, For example, the rabbit lacks the ready availability of genetic knockouts and sophis ticated tools for molecular analysis that are common in rodents. Furthermore, the benefit of high throughput animals must be weighed against the increased costs associated with purchasing, husbandry, and US Department of Agriculture records maintenance when compared to rodents.

5. Future directions and conclusions

Developing an in depth understanding of wound biofilms and potential therapeutics (Table 2), requires an *in vivo* model that can be utilized to understand the complex interactions that occur between bacteria and host. With the field of biofilm research continuing to grow, several animal models that address different aspects of wound biofilms have been developed, each with distinct advantages and disadvantages. As these models continue to be used and validated, researchers will be able to recognize the utility of one model over another based on the questions they hope to answer.

Table 2 – Tested, or potential, biofilm therapeutics.

Traditional wound care (sharp debridement, lavage, antibiotics)
Antimicrobial or silver impregnated dressings
D amino acids
Bacteriophages
Energy based (e.g., ultrasound) wound care devices
Virulence factor inhibitors (e.g., quorum sensing inhibition by RNAIII inhibiting peptide in *S aureus*)
Anti biofilm agents (e.g., lactoferrin aimed at decreasing surface attachment)

With a goal of clinically translatable results, further model development and improvement must continue to achieve a faithful representation of the biofilms seen in human chronic wounds. This may include modifications such as the introduction of polybacterial species within a single biofilm or the concurrent presence of systemic pathologies such as diabetes or venous insufficiency. In addition, mechanistic studies using bacterial mutants and/or targeted therapeutic agents in these models will improve our understanding of the *in vivo* pathways that dictate the resistance and defense mechanisms of biofilm phase bacteria. As the sophistication of *in vivo* biofilm modeling continues to grow, so will its practical impact on understanding and treating human chronic wounds, particularly when testing new hypotheses that will better help us elucidate the organization and persistence of biofilm communities in the susceptible human wound.

It is notable that most chronic wounds are not malignant and can persist in a state of coexistence with the patient for years. We hypothesize that the complex interactions between the multi species biofilm phenotype and the cutaneous wound likely involves a type of mutualism, whereby the bacteria employ a variety of decoy and signal manipulations to impede epithelialization, thereby prolonging the persistence of the wound "niche" in which they flourish and exist. Biofilms are also known to exhibit decreased levels of bacterial proliferation while triggering only a low grade inflammatory response from their host, further contributing to their maintenance within a wound [13]. Having likely evolved as a means to prevent their eradication from the wound habitat, it will be difficult to restore biofilm infected wounds to a healing phenotype without additional interventions. Through *in vivo* biofilm modeling, we aim to validate this hypothesis while testing those potential interventions that may have a significant impact on the future of chronic wound care.

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